Title: Diabetes induces endothelial dysfunction but does not increase neointimal formation in high fat diet fed C57BL/6J mice

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Online Supplement:

Materials and Methods:

Analytical procedures: Glucose levels were determined in fasted (4-6 hours) conscious mice from tail blood samples using a clinical glucometer (Glucometer Elite). Blood was collected from the retro-orbital venous plexus after isoflurane anesthesia for other biochemical measurements. Plasma was used for measurement of total cholesterol, triglyceride, and free fatty acids using enzymatic assays (Wako, Richmond, VA). Leptin and insulin were measured using ELISA assays from R&D (Minneapolis, MN) and from Alpco Diagnostics (Windham, NH).

For glucose tolerance tests, mice were fasted for 6 hours and were given an intraperitoneal injection of glucose (1mg/g body weight) in phosphate buffered saline (PBS). Blood glucose was determined from tail vein as described above at 0, 15, 30, 50, 90 and 120 min after injection.

Tissue collection: Mice were anesthetized with an intraperitoneal injection of ketamine and xylazine. The chest and peritoneal cavity were opened and the circulatory system was perfused via the left ventricle with phosphate buffered saline. Arteries were removed and processed for the various assays. For vascular studies, the left superficial femoral artery was removed and immediately placed in ice-cold physiologic salt solution (PSS). For arterial injury studies, arteries were isolated after paraformaldehyde fixation and processed as previously described ¹.

Vascular function studies: All reagents were acquired from Sigma Chemical (St.Louis, MO). Femoral arteries with intact endothelium and similar dimensions were mounted on a small vessel wire myograph (Danish MyoTechnology, Aarhus, Denmark) as described previously ².
were bathed in PSS at 37°C and aerated continuously with 5 % CO₂/ 95 % O₂ to achieve pH of 7.4. The arteries were set to a predetermined internal circumference, which has been shown to be the optimum caliber for studies of the peripheral vasculature. The startup protocol and evaluation of vessel viability was conducted as described previously. Concentration response curves were performed for potassium chloride, phenylephrine, norepinephrine, acetylcholine (endothelium dependent), sodium nitroprusside (endothelium-independent nitric oxide releasing agent). Due to differences in the arterial response to phenylephrine between the two diet groups, responses to vasodilator agents were determined following a stable contraction to KCl 50mM. Wall tension was expressed as milliNewton per millimeter (mN/mm) of artery length. Sensitivity to the agonist was expressed as the negative log of the effective concentration required to produce 50 % of maximum effect (-log EC50). Sensitivity was calculated from each concentration response curve by fitting the Hill equation using Prism (GraphPad Software, San Diego, CA). The operator was unaware of treatment groups during data collection.

Femoral artery wire injury: Endovascular wire injury was performed in the femoral arteries of mice as previously described. Briefly, a 0.25mm angioplasty guidewire was used to denude and dilate the femoral arteries of mice. Mice were sacrificed 4 weeks after arterial injury. Intimal and medial areas were measured on sections of femoral artery stained with combined masson elastin stain using computer aided morphometry.

Western Blotting: Mice were fasted for 4 hours and injected with PBS or human recombinant insulin 3.8mU/g mouse body weight in 200ul PBS via the retroorbital plexus. Arteries were harvested 4 minutes after injection. Protein was resolved on 4-15%SDS-PAGE reducing gels (Bio-rad). Protein was transferred to PVDF membranes (Bio-Rad), blocked in 5% milk/tris buffered saline (TBS), and probed with primary antibodies overnight. Membranes were
washed, incubated with appropriate secondary antibodies conjugated to horseradish peroxidase, washed in TBS-tween 0.05%, incubated with ECL reagent (Amersham Biosciences, Piscataway, NJ) and exposed to x ray film (Denville Scientific, South Plainview, NJ). Blots were stripped and reprobed with additional primary antibodies. Quantitative densitometry was performed using chemiDoc XRS imager using Quantity one-4.5.0 software (Bio-rad). Anti-akt antibodies were obtained from Cell Signaling (Beverly, MA). Anti eNOS antibodies were obtained from BD Transduction Laboratories (Franklin Lakes, NJ). HRP conjugated secondary antibodies were obtained from Pierce (Rockford, IL). For detection of eNOS dimers, we sought to minimize artifacts due to ex vivo disruption of eNOS dimers by performing low temperature (four degrees celcius) polyacrylamide electrophoresis as previously described. Pilot experiments demonstrated that incubation of the samples at 4, 25, and 37 degrees in the presence of SDS and 2.5% β-mercaptoethanol for up to 2 hours was not sufficient to denature the proteins in the arterial lysate, and eNOS protein did not migrate according to size on SDS-PAGE. When the protein lysates were heated to 65 degrees for 30 minutes in the presence of SDS and 2.5% β-mercaptoethanol, eNOS protein migrated according to its expected molecular weight. Although the heating procedure may have led to an underestimation of the absolute amount of eNOS dimers, all samples from both diet groups were processed simultaneously under the same conditions, and the relative differences between the diet groups persisted in all the experiments.

Nitrotyrosine immunohistochemistry: Sections from mice (n=3 from each diet group) were fixed with acetone and blocked with 8% goat serum in PBS for 1 hour. Sections were incubated with 1:100 dilution of rabbit anti-nitrotyrosine antibody (Upstate Technologies) in 4% goat serum overnight at 4 degrees. After washing, alexa 488 conjugated anti-rabbit antibody (Molecular Probes) was applied for 30 minutes, sections were washed and incubated with hoechst dye33258
(Molecular Probes) and coverslipped. Fluorescence images were acquired using fixed exposure times using a CCD camera attached to a Zeiss Axiovert inverted microscope. Negative and positive control sections were preincubated with 3-nitrotyrosine (Sigma), or a peroxynitrite solution (Upstate), respectively, to assure specificity of staining.

**Statistics:** Data are represented as mean±standard error of the mean (sem). Significance was assessed by two-tailed Student’s t-test for parametric data at p<0.05. Bonferroni post tests were utilized after 2 way analysis of variance (ANOVA) to detect differences between groups. Statistical tests were performed using Prism software (GraphPad).
Online Table I

<table>
<thead>
<tr>
<th>Diet</th>
<th>Period of feeding</th>
<th>Weight</th>
<th>Leptin</th>
<th>Glucose</th>
<th>Insulin</th>
<th>TC</th>
<th>TG</th>
<th>FFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>chow</td>
<td>9 weeks</td>
<td>32±1(17)</td>
<td>4±1(10)</td>
<td>135±7(10)</td>
<td>0.28±0.04(10)</td>
<td>79±4(7)</td>
<td>46±2(7)</td>
<td>0.59±0.04(10)</td>
</tr>
<tr>
<td>high fat</td>
<td>9 weeks</td>
<td>39±1(30)*</td>
<td>56±5(20)*</td>
<td>226±9(20)*</td>
<td>2.5±0.36(25)*</td>
<td>139±6(10)*</td>
<td>14±1(11)*</td>
<td>0.47±0.06(10)</td>
</tr>
</tbody>
</table>

Data expressed as mean ± standard error of the mean with the number of mice in parentheses. *p<0.0001
References:


